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Thermodynamics of phosphorylcholine and lysophosphatidylcholine binding to the major protein of bovine seminal plasma, PDC-109

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Abstract PDC-109 binds to sperm plasma membranes by specific interaction with choline phospholipids and induces cholesterol efflux, a necessary event before capacitation – and subsequent fertilization – can occur. The binding of phosphorylcholine (PrC) and lysophosphatidylcholine (Lyso-PC) with PDC-109 was investigated by monitoring the ligand-induced changes in the absorption spectrum of PDC-109. At 20 °C, the association constants (K_a), for PrC and Lyso-PC were obtained as 81.4 M^{-1} and 2.02×10^4 M^{-1}, respectively, indicating that the binding of Lyso-PC to PDC-109 is 250-fold stronger than that of PrC. From the temperature dependence of the K_a values, enthalpy of binding (ΔH^0) and entropy of binding (ΔS^0), were obtained as −79.7 and −237.1 J mol^{-1} K^{-1} for PrC and −73.0 kJ mol^{-1} and −167.3 J mol^{-1} K^{-1} for Lyso-PC, respectively. These results demonstrate that although the binding of these two ligands is driven by enthalpic forces, smaller negative entropy of binding associated with Lyso-PC results in its significantly stronger binding.

Keywords: Bovine seminal plasma proteins-A1/A2; Cholesterol efflux; Choline phospholipid; Binding enthalpy; Binding entropy

1. Introduction

In mammals, freshly ejaculated spermatozoa cannot fertilize the egg until they undergo a series of biochemical and ultrastructural changes that occur during their residence in the female genital tract. These multifaceted changes are referred to as capacitation, a process that is poorly understood at the molecular level [1,2]. Studies on a number of species have shown that the seminal plasma contains several proteinaceous factors that play a crucial role in priming the spermatozoa for fertilization. The bovine seminal plasma contains four acidic proteins – designated BSP-A1, BSP-A2, BSP-A3 and BSP-30-kDa – that bind the spermatozoa. Collectively they are referred to as bovine seminal plasma proteins or as *BSP proteins* [3,4]. BSP-A1 and BSP-A2 have the same primary structure and differ only in the degree of glycosylation and their mixture is also referred to as PDC-109 [5].

PDC-109 is the major protein of the bovine seminal plasma and is present at a concentration of ca. 15–25 mg/ml [6]. Its 109-residue polypeptide chain is made up of two fibronectin type-II (FnII) domains, preceded by a 23-residue *N*-terminal stretch, which is rich in acidic amino acids [5,7,8]. Recent biochemical and biophysical studies suggest that PDC-109 is a multifunctional protein, with at least two different types of physiologically significant binding interactions [cf. 9]. Single-crystal X-ray diffraction studies have shown that each FnII domain can bind one choline phospholipid molecule by its specific interaction with the phosphorylcholine moiety and that the two binding sites are on the same face of the protein [10]. The interaction of PDC-109 with sperm plasma membranes results in an efflux of cholesterol and choline phospholipids, referred to as *cholesterol efflux*, which appears to be an important step in the capacitation process, which in turn is a necessary event before fertilization can occur [11,12]. PDC-109 also interacts with fucosylated oligosaccharides present on the oviductal epithelium in cow [13], and this has been postulated to be responsible for the maintenance of oviductal sperm reservoir [14].

In view of the above, it is important to investigate the binding of different ligands to PDC-109 in order to understand its role in the fertilization process. In previous spin-label EPR studies it was shown that upon binding to dimyristoylphosphatidylcholine (DMPC) membranes, PDC-109 penetrates into the hydrophobic interior of the membrane and that it also recognizes other phospholipids such as phosphatidylglycerol and phosphatidylserine, albeit weakly [15]. Binding of PDC-109 decreases phospholipid mobility and abolishes the lipid chain-melting phase transition [15–17]. Presence of cholesterol modulates the binding by increasing the association of different phospholipids and sterol probes with the protein, although the relative selectivity for individual lipid species was not significantly affected [18]. Surface plasmon resonance (SPR) studies have shown that the binding of PDC-109 to phosphatidylcholine (PC) membranes is due to a combination of faster association rate constant and a slower dissociation rate constant, as compared to other phospholipids [19]. In the present study the binding of PrC and Lyso-PC to PDC-109 has been investigated by absorption spectroscopy. The thermodynamic parameters associated in these interactions have been delineated by performing the binding studies at different temperatures. The results indicate that although the binding of both PrC and Lyso-PC to PDC-109 is driven by enthalpic forces, binding of PDC-109 to Lyso-PC is 250-fold stronger than its interaction with PrC due to a significantly smaller negative entropic contribution associated with the former.

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2. Materials and methods

2.1. Materials

Phosphorylcholine chloride (Ca^{2+} salt) and Tris base were obtained from Sigma (St. Louis, MO, USA). Lyso-PC from egg yolk, which contains primarily palmitoyl and stearoyl chains, was a product of Avanti Polar Lipids (Alabaster, AL, USA). Sephadex G-50 (superfine) and DEAE Sephadex A-25 were purchased from Pharmacia (Uppsala, Sweden).

2.2. Purification of PDC-109

PDC-109 was purified from bovine seminal plasma from healthy and reproductively active bulls by gel filtration on Sephadex G-50 followed by affinity chromatography on DEAE Sephadex A-25 [cf. 15,18]. Purity of the protein was assessed by SDS–PAGE using 10% or 12 % acrylamide gels [20], where it moved as two closely spaced bands of $M_r \sim 13$ kDa, corresponding to the glycosylated and unglycosylated forms [21]. Concentration of PDC-109 was estimated from its extinction coefficient of 2.5 for 1 mg/ml concentration at 280 nm [21].

2.3. Binding of phosphorylcholine and Lyso-PC to PDC-109

Binding of PrC and Lyso-PC to PDC-109 was investigated by absorption spectroscopy. All experiments were carried out in 50 mM Tris–HCl buffer, pH 7.4, containing 0.5 M NaCl, 5 mM EDTA and 0.025% sodium azide (TBS). Absorption spectra were recorded on a Shimadzu UV-3101PC UV–Vis–NIR double-beam spectrophotometer using 1.0-cm path-length cells. Temperature was maintained constant (±0.5 °C) by means of a Peltier device. Titrations were performed by adding small aliquots of the ligands from stock solutions (200 mM PrC, 2.0 mM Lyso-PC) to both the sample and reference cuvettes. The concentration of PDC-109 in different titrations ranged between 0.32 and 0.44 mg/ml. Spectra were recorded after an equilibration period of 2 min following each addition. All titrations were performed at least two times and the average values are reported.

2.4. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded at 25 °C on a Jasco-J-810 spectropolarimeter at a scan speed of 20 nm/min using 0.1-cm path-length cylindrical quartz cells. Far-UV and near-UV spectra were recorded at PDC-109 concentration of about 0.16 mg/ml and 0.64 mg/ml, respectively. Data were collected with a response time of 2 s and a slit width of 1 nm. Each spectrum reported was the average of 20 consecutive scans from which buffer scans, recorded under the same conditions, were subtracted. The observed ellipticities were converted to mean residue ellipticities (Θ) using a mean molecular mass/residue of 117 [5].

3. Results and discussion

Previous binding studies have demonstrated that PDC-109 specifically recognizes choline phospholipids such as phosphatidylcholine and sphingomyelin, among the different types of lipids tested [22]. Affinity chromatographic experiments in which PrC was successfully used as the eluting ligand showed that the protein specifically recognizes the choline moiety in choline phospholipids [23]. PDC-109, which exists as a polydisperse aggregate in solution, is converted into dimers upon binding of PrC [24]. The X-ray structure of the PDC-109/PrC complex showed that each FnII domain binds one molecule of PrC [10]. Although it is known from earlier reports that both PrC and Lyso-PC bind to PDC-109 and that the binding affinity for the interaction of PDC-109 with Lyso-PC is higher than the affinity of its interaction with PrC [22–26], the association constants and thermodynamic forces that govern the interaction of choline-containing ligands to this protein were not known. In this study we have investigated the binding of PrC and Lyso-PC to PDC-109, in order to obtain the association constants that characterize the respective binding reactions and to delineate the thermodynamic forces that control their interaction.

When PDC-109 was titrated with PrC, small but reproducible changes were observed in the protein absorption spectrum. Difference spectra displayed an increase in the absorption intensity in the wavelength range of ca. 250–300 nm, with maximum change in absorption around 289 nm and a second peak centered around 280 nm, signifying the perturbation of aromatic side chains of Tyr and Trp (not shown). Titration of PDC-109 with choline chloride led to very small changes in the absorption spectrum of the protein, precluding the analysis of the titration data to obtain the association constant. On the other hand, titration of the protein with Lyso-PC resulted in a considerably larger increase in the absorption intensity of the protein in the same spectral region (Fig. 1A). Further, as seen in the difference spectra (Fig. 1B), the change in the absorption intensity at 280 nm was considerably larger than that at 289 nm, indicating that Tyr residues are perturbed more in

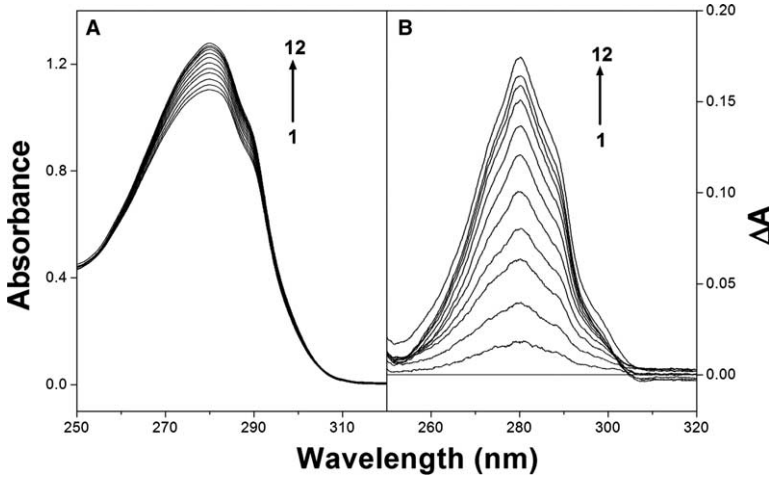

Fig. 1. Absorption titration of PDC-109 with Lyso-PC. (A) Absorption spectra of PDC-109 alone (spectrum 1) and PDC-109 in the presence of different concentrations of Lyso-PC (spectra 2–12). (B) Difference absorption spectra obtained by subtracting the spectrum of PDC-109 alone from the spectra of PDC-109 obtained in the presence of different concentrations of Lyso-PC. The difference spectra with increasing intensities were obtained in the presence of increasing concentrations of Lyso-PC.

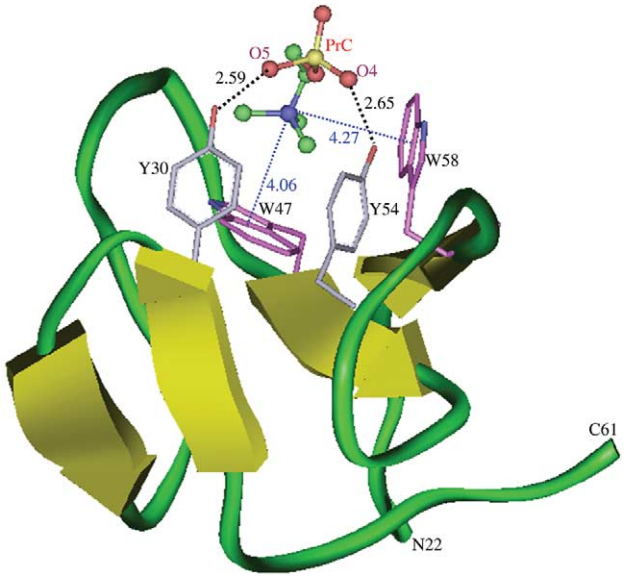

Fig. 2. Structure of a FnII domain of PDC-109 with bound PrC molecule. The structure was generated using the Insight II software (Accelrys Inc.) from the coordinates in pdb (id=1h8p). The backbone is shown as a ribbon and the side chains of Y30, Y54, W47 and W58, which interact with the ligand are shown as sticks. Hydrogen bonds between the hydroxyls of Y30 and Y54 and the phosphate oxygens are indicated by black dotted lines. The cation–π interaction of the quaternary ammonium group with W47 and W58 are indicated by blue dotted lines with the distances between the quaternary nitrogen and the centroid of the indole moiety. Distances are shown in Å. See text for details.

the case of Lyso-PC binding. These observations are consistent with the three-dimensional structure of PDC-109/PrC complex, obtained from single-crystal X-ray diffraction studies [10], where it was observed that binding of PrC is guided by a cation–π interaction between the quaternary ammonium group and the indole side chain of a core tryptophan residue in the ligand binding site, with additional hydrogen bonding interactions between the phosphate group of PrC and side chains of one or two tyrosine residues (see Fig. 2). Although the crystal structure suggested that the quaternary ammonium group interacts with the indole side chain of Trp47, in solution a similar interaction with the indole moiety of Trp58 is also quite probable; such an interaction requires only a rotation around the C–C bond between the two methylene units of phosphorylcholine.

Binding of PrC and Lyso-PC to PDC-109 was monitored by following changes in the absorbance at 289 and 280 nm, respectively. Typical binding curves for the association of PrC and Lyso-PC with PDC-109 are shown in the insets of Figs. 3A and B, respectively. These binding curves show that the change in the absorption intensity decreases with increasing ligand concentration, displaying saturation behavior. At the highest concentrations of the ligands used in the binding titrations performed at 20 °C (binding curves shown in the insets of Figs. 3A and B), the ratios PrC/PDC-109 and Lyso-PC/PDC-109 (monomer) are 656 and 4.5 and clearly indicate that Lyso-PC exhibits a significantly higher binding strength as compared to PrC. These results are in agreement with the observations of Müller et al. [25], who investigated the interaction of Lyso-PC and PrC with PDC-109 at 30 °C by fluorescence spectroscopy.

In order to obtain the association constants, K_a the binding data were analysed according to the expression [27]:

$$\mathrm{Log}\{\Delta A/(A_c - A_\infty)\} = \log K_a + \log[L]_f \quad (1)$$

where ΔA is the change in absorbance at any point of the titration, A_c is the corresponding absorption intensity of the protein, A_∞ is the absorption intensity of the protein that is fully saturated with the ligand and $[L]_f$ the free ligand concentration, is given by:

$$[L]_f = [L]_t - \{(\Delta A/\Delta A_\infty) \cdot [P]_t\} \quad (2)$$

where $[P]_t$ is the total protein concentration, $[L]_t$ is the total ligand concentration and ΔA_∞ is the change in absorbance at saturation binding. Because the three-dimensional structure of PrC/PDC-109 complex [10] has clearly shown that each molecule of PDC-109 binds two molecules of PrC, in the analysis of the titration data the protein concentration was taken as twice the concentration of PDC-109 monomer, i.e., the concentration of the FnII domains.

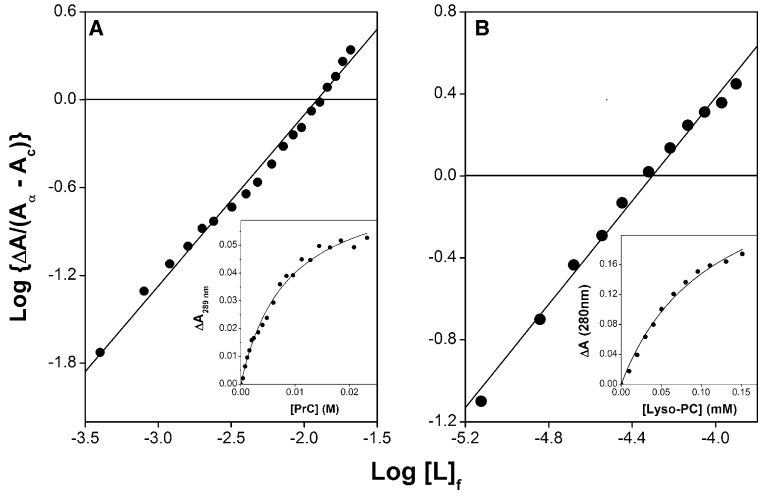

Fig. 3. Analysis of the absorption titration with PrC (A) and Lyso-PC (B). The titration data were analysed according to [25]. The insets show binding curves of ΔA versus $[L]_f$. See text for details.

Eq. (1) shows that the X-intercept of a plot of $\log\{\Delta A/(A_c - A_\infty)\}$ versus $\log[L]_f$ will yield pK_a for the association of PrC (or Lyso-PC) with PDC-109. Representative plots of this type corresponding to the interaction of PrC and Lyso-PC with PDC-109 at 20 °C are given in Figs. 3A and B, respectively. From the X-intercepts of these plots the K_a values characterizing the binding of PrC and Lyso-PC to PDC-109 were obtained as 81.4 M^{-1} and 2.02×10^4 M^{-1}, respectively.

It is seen that the data shown in Figs. 3A and B exhibit linear dependence within the range of experimental error, suggesting that despite the minor differences in the mode of binding of PrC to the different FnII domains of the protein as observed in the PrC/PDC-109 complex crystal structure [10], the binding strength for the interaction of PrC with the different FnII domains of PDC-109 is most likely comparable. In other words, the affinities with which different FnII domains of the PDC-109 dimer bind PrC appear to be nearly the same.

In order to obtain the thermodynamic parameters associated with the interaction of PrC and Lyso-PC, binding titrations were performed at different temperatures. The association constants were then obtained by analyzing the titration data as described above and the values obtained at different temperatures are listed in Table 1. It is clear from the values presented in the table that the association constants (K_a) decrease with increase in temperature for both PrC and Lyso-PC.

From the K_a values, changes in the Gibbs free energies have been calculated using Eq. (3) as $\Delta G^0 = -10.7$ and -24.3 kJ mol^{-1} for the interaction of PDC-109 with PrC and Lyso-PC, respectively.

$$\Delta G^0 = -RT \ln K_a \quad (3)$$

The thermodynamic parameters, enthalpy of binding (ΔH^0) and entropy of binding (ΔS^0) associated with the interaction of PrC and Lyso-PC were obtained by means of van't Hoff plots (Fig. 4) according to the expression:

$$\ln K_a = (-\Delta H^0/RT) + (\Delta S^0/R) \quad (4)$$

The enthalpy and entropy of binding for PrC were obtained as $\Delta H^0 = -79.7$ kJ mol^{-1} and $\Delta S^0 = -237.1$ J mol^{-1} K^{-1}, whereas the corresponding values for the binding of Lyso-PC were determined to be $\Delta H^0 = -73.0$ kJ mol^{-1} and $\Delta S^0 = -167.3$ J mol^{-1} K^{-1}.

It is instructive to compare the K_a values and thermodynamic parameters obtained for the association of PrC and Lyso-PC with PDC-109 (Table 1). The K_a value for the Lyso-PC/PDC-109 interaction obtained at 20 °C is 2.02×10^4 M^{-1}, which is about 250-fold higher than the value of 81.4 M^{-1} obtained for the association of PrC with PDC-109 at the same temperature. A comparison of the thermodynamic parameters obtained with PrC and Lyso-PC clearly shows that although the binding of PrC is associated with a slightly larger (negative) change in enthalpy as compared to Lyso-PC, the smaller negative contribution from entropy of binding overrides this difference, resulting in a much stronger association of Lyso-PC with PDC-109.

Table 1
Association constants, K_a, determined from the absorption titrations at different temperatures for the binding of phosphorylcholine and Lyso-PC to PDC-109

T (°C)	Phosphorylcholine K_a (M^{-1})	Lyso-PC $10^{-3} \times K_a$ (M^{-1})
15	99.0 (±1.1)	–
20	81.4 (±0.1)	20.2 (±0.4)
25	33.2 (±0.7)	11.2 (±0.9)
30	28.3 (±0.5)	5.6 (±0.7)
35	11.8 (±1.3)	5.3 (±0.2)

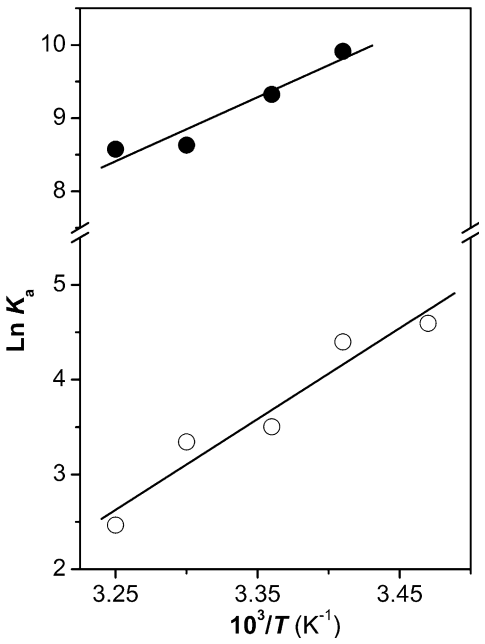

Fig. 4. van't Hoff plots for the interaction of phosphorylcholine (○) and Lyso-PC (●) with PDC-109.

Previous CD spectroscopic studies have shown that PrC binding leads to notable changes in the secondary and tertiary structure of PDC-109 [24]. However, the effect of Lyso-PC binding on the structure of this protein was not known. In order to investigate this and to compare the effect of these two ligands on the structure of PDC-109 under similar conditions, we have recorded the CD spectra of PDC-109 in TBS (pH 7.4). The far-UV and near-UV CD spectra of PDC-109 alone and in the presence of PrC and Lyso-PC are shown in Figs. 5A and B, respectively. Consistent with the previous reports, the far-UV CD spectrum of PDC-109 is characterized by a broad positive band centered at ca. 225 nm, which appears to be due to the unusually high content of aromatic amino acids and disulfide bonds in this protein [24]. Analysis of the far UV CD spectra to obtain secondary structure composition was not possible due to the lack of appropriate protein reference data set [5,24]. Binding of either PrC or Lyso-PC results in a considerable increase in the intensity of this band with the magnitude of change associated with the binding of Lyso-PC being larger. These results, together with the results of absorption titrations presented above, which showed larger changes in the difference spectra in the presence of Lyso-PC, clearly indicate that the binding of Lyso-PC induces larger perturbations in the secondary structure of PDC-109. The near-UV CD spectra of PDC-109 exhibit similar trends, with two overlapping positive bands at 282 and 289.5 nm, which become more intense in the presence of PrC with practically no shift in the band position. In the presence of Lyso-PC also two closely spaced and overlapping bands are seen; however, these bands are slightly blue shifted and occur at 280 and 286.5 nm. Although X-ray studies

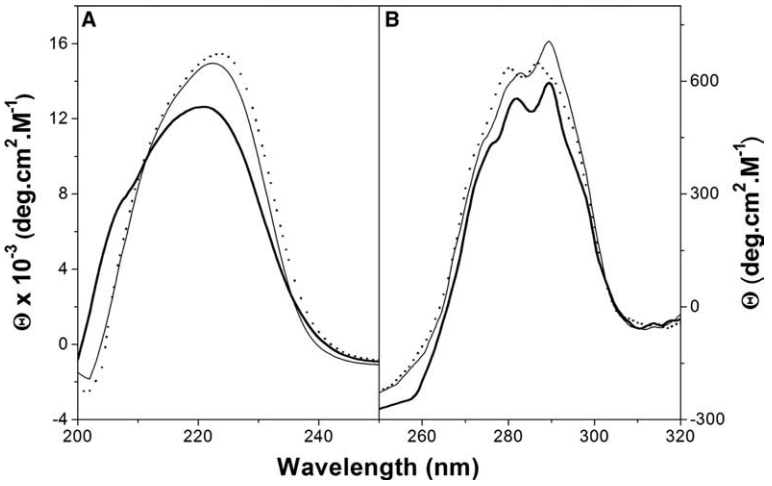

Fig. 5. CD spectra of PDC-109 in the absence and in the presence of PrC and Lyso-PC. (A) Far-UV region and (B) near-UV region. (——) PDC-109 alone, (——) PDC-109 + 20 mM PrC, (----) PDC-109 + 0.2 mM Lyso-PC. See text for details.

have shown that binding of PrC leads to conformational changes in the polypeptide backbone of PDC-109 [10], changes observed in the far and near-UV CD spectra upon binding of PrC or Lyso-PC could arise in part due to the changes in the quaternary structure of the protein, as it has been demonstrated earlier that this protein, which exists as a polydisperse aggregate in solution, is converted to the dimeric form upon ligand binding [24].

Our previous surface plasmon resonance studies have shown that association of PDC-109 with DMPC/cholesterol mixtures is entropically favored, with binding enthalpy (ΔH^0) and entropy (ΔS^0) values of 7.08 kJ mol^{-1} and 164.36 J mol^{-1} K^{-1} respectively (19). Comparing the thermodynamic parameters obtained for the interaction of PrC and Lyso-PC with these values indicates that going from PrC to Lyso-PC to DMPC, the binding enthalpy becomes progressively less favorable, whereas the entropy of binding becomes increasingly favorable. However, the net result is that the free energy of binding becomes more favorable, resulting in a stronger binding of DMPC as compared to Lyso-PC, which in turn binds more strongly than PrC. Although several possibilities can be suggested for the above changes in the binding enthalpy and binding entropy for the interaction of the above three ligands with PDC-109, it is important to obtain structural information on the complexes of PDC-109 with Lyso-PC and diacyl PC in order to gain molecular level understanding on the factors leading to the energetic changes with respect to the binding of these ligands to PDC-109.

Results from a number of studies indicate that PDC-109 exhibits an obligatory requirement for choline-containing lipids in order to bind to lipid membranes [15,19,22,23,25]. Indeed, the X-ray structure of PrC/PDC-109 complex [10] clearly shows that specific recognition of the phosphorylcholine moiety by each FnII domain of the protein is mediated by a cation–π interaction between the quaternary ammonium group and a core tryptophan and several hydrogen bonds between the phosphate group of the ligand and the hydroxyl groups of tyrosine residues (Fig. 2). The present results clearly show that despite the obligatory requirement of the choline moiety for PDC-109 to bind to lipid membranes, binding of phosphorylcholine to this protein is quite weak, with an association constant of 81.4 M^{-1} at 20 °C. The association becomes 250-fold stronger by the attachment of the glycerol backbone and the acyl chain in Lyso-PC, with a K_a of 2.02×10^4 M^{-1} at the same temperature. Comparison with the SPR results shows that the strength of binding of the diacyl lipid, DMPC ($K_a = 2.1 \times 10^7$ M^{-1}) is further increased by three orders of magnitude [19]. These results show that the additional interactions with the acyl chain(s) further stabilize the binding of Lyso-PC and DMPC with PDC-109. However, phospholipids with other head groups are very poorly recognized [15,19,22,25], clearly indicating the requirement of both phosphorylcholine moiety and the hydrophobic acyl chain region of the choline phospholipids for the optimal interaction of PDC-109 with sperm cell membranes. Phosphatidylcholine, which is the major lipid of the sperm plasma membrane, thus appears to have been chosen for this regulatory step in the sperm cell maturation prior to fertilization.

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